

WHAT IS CLAIMED IS:

- 1 1. A method for engineering a cell to produce an increased amount of hydrogen
2 comprising:
 - 3 (a) providing a mutagenized nucleic acid sequence derived from a first gene that
4 encodes a protein involved in a hydrogen production pathway;
 - 5 (b) transforming a cell with said mutagenized nucleic acid sequence; and
 - 6 (c) screening or selecting the cell for an increased amount of hydrogen.
- 7
- 8 2. The method of claim 1, wherein a plurality of mutagenized nucleic acid sequences are
9 used to transform a population of cells, followed by the screening or selecting.
- 10
- 11 3. The method of claim 1, wherein the first gene is selected from the group that encodes
12 ferredoxin, catalase, isoamylase, malate dehydrogenase, 14-3-3 protein, enolase, aldolase,
13 ribosomal protein S8, ribosomal protein L17, ribosomal protein S18, ribosomal protein L37,
14 ribosomal protein L12, ribosomal protein S15, iron-hydrogenase, nickel-iron hydrogenase,
15 and components of the photosystem I, photosystem II, light harvesting antenna and
16 cytochrome b₆-f complexes.
- 17
- 18 4. The method of claim 3, wherein the first gene encodes an iron-hydrogenase.
- 19
- 20 5. The method of claim 4, wherein at least one amino acid from the segment
21 X¹X²X³FX⁴X⁵X⁶GGVMEAAX⁷R or the segment ADX⁸TIX⁹EE is substituted by a different
22 amino acid in the protein encoded by the first gene to generate the mutagenized nucleic acid
23 sequence.
- 24
- 25 6. The method of claim 5, wherein the mutagenized nucleic acid sequence is generated
26 by gene reassembly.
- 27
- 28 7. The method of claim 5, wherein the mutagenized nucleic acid sequence is generated
29 by site-directed mutagenesis.
- 30
- 31 8. The method of claim 5, wherein an amino acid that is substituted for the at least one
32 amino acid has a side chain of higher molecular weight than the side chain of the at least one
33 amino acid.

- 34
- 35 9. The method of claim 5, wherein saturation mutagenesis is performed on the at least
- 36 one amino acid.
- 37
- 38 10. The method of claim 5, wherein the mutagenized nucleic acid sequence is generated
- 39 by a mutagenesis method described in U.S. Patents selected from the group consisting of
- 40 5,537,776; 5,965,408; 6,171,820; 6,174,673; 6,238,884; 6,326,204; 6, 344,328; 6,352,842;
- 41 6,358,709; 6361,97; 6,368,798; 6,440,668; 6537,776; and 6,605,449.
- 42
- 43 11. The method of claim 6, wherein the gene reassembly is performed using nucleic acid
- 44 molecules that encode proteins of SEQ ID NOs: 1-112 or segments thereof.
- 45
- 46 12. The method of claim 4, wherein the mutagenized nucleic acid sequence encodes an
- 47 iron hydrogenase protein that functionally interacts with a ferredoxin protein in the cell.
- 48
- 49 13. The method of claim 1, wherein the screening or selecting occurs in the presence of
- 50 oxygen at a concentration selected from the ranges comprising more than 0.5%, more than
- 51 5.0%, more than 10%, more than 15%, approximately 21%, more than 21%, more than 25%,
- 52 more than 30% or more than 35% oxygen.
- 53
- 54 14. The method of claim 1, wherein the mutagenized nucleic acid sequence is operably
- 55 linked to a promoter that is activated by light.
- 56
- 57 15. The method of claim 1, wherein the mutagenized nucleic acid sequence is generated
- 58 by gene reassembly.
- 59
- 60 16. The method of claim 1, wherein the cell is a green algae species.
- 61
- 62 17. The method of claim 1, wherein cell is of the genus *Chlamydomonas*.
- 63
- 64 18. The method of claim 1, further comprising the steps of;
- 65 (a) identifying a first independent transformant which produces an increased amount
- 66 of hydrogen from step (c) of claim 1;

67 (b) recovering the mutagenized nucleic acid sequence from the independent
68 transformant;
69 (c) further mutagenizing the recovered mutagenized nucleic acid sequence to create a
70 new library of mutagenized nucleic acid sequences;
71 (d) transforming cells with the new library of mutagenized nucleic acid sequences;
72 and
73 (e) screening or selecting for a new independent transformant from the new library
74 that generates an increased amount of hydrogen compared to the first independent
75 transformant.

76
77 19. The method of claim 18 wherein the mutagenized nucleic acid sequences are generated
78 by gene reassembly.

79
80 20. The method of claim 18, wherein a plurality of mutagenized nucleic acid sequences
81 are recovered from a plurality of independent transformants which produce an increased
82 amount of hydrogen from step (c) of claim 1, and wherein the plurality of mutagenized
83 nucleic acid sequences are subjected to gene reassembly to generate the new library.

84
85 21. The method of claim 1, wherein the screening or selecting occurs by culturing cells in
86 liquid growth media.

87
88 22. The method of claim 21, wherein the growth media is a photoautotrophic growth-
89 requiring minimal media.

90
91 23. The method of claim 1, wherein the screening or selecting occurs in a non-transparent
92 culture container.

93
94 24. A method according to claim 1, wherein the mutagenized nucleic acid sequence is
95 operably linked to a promoter that is constitutively activated.

96
97 25. The method of claim 15, wherein the mutagenized nucleic acid sequence is obtained
98 by subjecting nucleic acid sequences that encode proteins that are expressed when cells are
99 exposed to conditions more conducive to the generation of hydrogen to gene reassembly,

100 wherein the proteins are naturally encoded by genes in organisms from more than one
101 species.
102

103 26. The method of claim 19, wherein the proteins are iron hydrogenases or nickel-iron
104 hydrogenases.
105

106 27. The method of claim 1, further comprising repeating the steps of claim 1 using a
107 second gene distinct from the first gene.
108

109 28. The method of claim 27, further comprising:
110 (a) mating at least one cell of a strain containing a mutagenized form of the
111 first gene:
112 i. wherein the at least one cell is identified by the screening or
113 selecting; or
114 ii. wherein the at least one cell is derived through mating from a cell
115 identified by the screening or selecting;
116 to at least one cell of a distinct strain containing a mutagenized form of the
117 second gene:
118 iii. wherein the at least one cell is identified by the screening or
119 selecting; or
120 iv. wherein the at least one cell is derived through mating from a cell
121 identified by the screening or selecting; and
122 (b) screening or selecting for a progeny cell that produces an increased
123 amount of hydrogen compared to any parent cell.
124

125 29. A method of hydrogen production, comprising:
126 (a) placing cell containing a mutagenized nucleic acid sequence corresponding to a
127 gene that is involved in a hydrogen production pathway into liquid culture media
128 or on to solid culture media, wherein the mutagenized nucleic acid sequence is
129 operably linked to a transcriptional promoter sequence;
130 (b) culturing said transformed cell under conditions sufficient to stimulate
131 transcription of said mutagenized nucleic acid sequence(s); and
132 (c) collecting an evolved gas.
133

134 30. The method of claim 29, wherein the culture media is photoautotrophic growth
 135 requiring media.
 136

137 31. A method of multiparental mating of microbes that mate in response to a stimulus,
 138 comprising:
 139 (a) providing a cell from each of 3 or more strains of microbes capable of
 140 mating to each other in culture medium;
 141 (b) providing the stimulus;
 142 (c) allowing cells to mate and produce progeny;
 143 (d) allowing the progeny cells to achieve sexual reproduction capability;
 144 (e) providing the stimulus at least one more time; and
 145 (f) screening or selecting the further progeny for a desired phenotype.
 146

147 32. The method of claim 31, wherein the microbes are green algae and the stimulus is the
 148 removal of nitrogen from the media and illumination by light comprising a wavelength
 149 between about 0.42-0.52 micrometers.
 150

151 33. The method of claim 32, wherein the green algae are of the *Chlamydomonas* genus.
 152

153 34. The method of claim 33, wherein the species is selected from the group comprising
 154 *reinhardtii*, *eugametos*, *incerta*, and *moewusii*.
 155

156 35. The method of claim 31, wherein the stimulus is interruption of exponential growth in
 157 continuous light with a reduction in light, followed by addition of light.
 158

159 36. The method of claim 35, wherein the reduction in light occurs for a period selected
 160 from the group consisting of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more than 12 hours.
 161

162 36. The method of claim 31, wherein the microbes are of the *Scendesmus* genus and the
 163 stimulus is the addition of chromium to the culture media.
 164

165 37. The method of claim 31, wherein the desired phenotype is hydrogen production.
 166

167 38. The method of claim 31, wherein nucleic acid exchange occurs between only two
168 parental cells at a time during the mating process.
169